# Erythrocyte glutathione S transferase as a marker of oxidative stress at birth.

# Erythrocyte glutathione S transferase as a marker of oxidative stress at birth

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**Abstract**

***Aims*—To determine the level of oxidative stress and cell damage as a result of exposure to $O_2$ at birth.**

***Methods*—Using glutathione S transferase (GST) as an indicator of oxidative stress, GST activity in cord blood was compared with that in samples taken three hours after birth. Twenty four prematurely born infants and eight full term infants were studied. To test whether stronger effects occur under less favourable conditions, the neonates were divided in three groups: healthy premature; sick premature; and healthy full term infants.**

***Results*—GST activity three hours after birth was significantly decreased compared with that at birth in all three groups tested. There were no significant differences in the magnitude of this effect among the three groups.**

***Conclusions*—These results indicate that a sudden increase in oxygenation exposes the neonate to oxidative stress. Measurement of GST activity might be useful for the evaluation of protective treatment in trials considering antioxidant strategies.**

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Keywords: glutathione S transferase; oxidative stress; birth; prematurity

Birth exposes the neonate to a higher $pO_2$ than experienced in utero. The sudden increase in alveolar oxygen concentration and arterial $pO_2$ after delivery increases the formation of reactive oxygen species in the lung[1] and other organs. Free oxygen radicals seem to have a role in mediating tissue damage in hyperoxia and reperfusion after ischaemia,[2] in inflammatory processes,[3] and after circulatory shock.[4] When the antioxidant capacity of the neonate is inadequate to oppose the increased formation of reactive oxygen species after birth, this may result in an oxidative stress and cell damage.

The main glutathione S transferase (GST) isoenzyme in human erythrocytes, with activity towards the common substrate 1-chloro-2,4-dinitro-benzene (CNDB), belongs to the π-class.[5] Rat GST of this class can be deactivated by oxidation of a cysteine (47-Cys) near the active centre.[6] We have already shown that inhibition of erythrocyte GST from human adults can be used in vitro to indicate oxidative stress after treatment with hydroxylamines,[7] in vivo after intensive exercise,[8] and in coal workers' pneumoconiosis.[9] GST activity with the same catalytic properties as the enzyme form in adult erythrocytes, is present after 12 weeks of gestation in fetal erythrocytes.[10] In contrast to catalase and selenium dependent glutathione peroxidase, GST activity in erythrocytes is inversely related to increasing gestational age, and adult values are lower than any of the neonatal values.[11]

On the assumption that the glutathione S transferase enzyme in neonatal red blood cells has the same properties as those of adult erythrocytes, it is to be expected that this enzyme is also vulnerable to oxidative stress. When strong oxidative stress conditions occur at birth, the activity of such a vulnerable enzyme could decrease. The extent of that decrease could be even greater under more extreme oxidative stresses.

To test GST sensitivity in neonates, we compared the in vitro effects of hydrogen peroxide on GST activity in cord red blood cells and adult red blood cells. To verify the actual decreases in GST activity at birth, we compared GST values in cord blood with those in blood samples taken three hours after birth. To verify whether stronger effects occur under less favourable conditions, the neonates were divided in three groups: healthy prematurely born; sick prematurely born; and healthy full term infants.

## Methods

Ethical approval for the study was granted by the Ethical Committee of the Academic Hospital Maastricht and St Maartens Gasthuis Venlo. Informed consent was obtained from all the parents and adult volunteers.

Twenty four prematurely born infants (gestational age 24 to 33 weeks) and eight full term infants were enrolled into the study. Infants with major congenital abnormalities were excluded. In the prematurely born infant group, seven infants required intensive care during the first three hours of life. All of them were intubated directly after birth. They had respiratory distress syndrome ( n=6), birth asphyxia (n=1), early onset sepsis (n=3), cardiovascular instability requiring intravascular volume expansion and adrenergic drugs (n=3) and persistent pulmonary hypertension of the newborn requiring nitric oxide (n= 1). These infants were classified as "sick." The seventeen remaining premature infants required no specific support and were classified as "healthy." None of the eight full term infants required specific support. All infants were treated according to hospital procedures at that time. The oxygen saturation was kept between 86% and 93%.

Venous blood samples from four healthy adult volunteers and a blood sample of a segment of the umbilical cord of five full term infants were collected for the in vitro incubations. Venous

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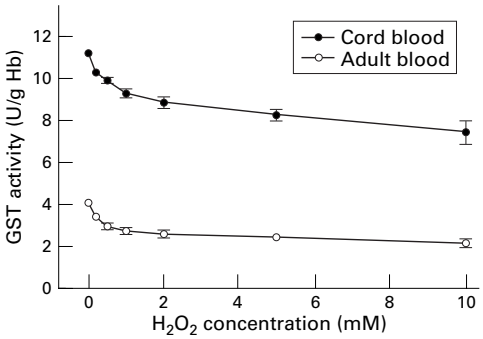


*Figure 1 Changes in erythrocyte GST activity in adult (n=47) and cord blood samples (n=5) after treatment with hydrogen peroxide for 1 hour at 37° C. Error bars represent SEM differences between sample value and control value.*

blood samples from adult volunteers were drawn in 10 ml vacuum tubes containing ethylene diamine-tetra acetic acid (EDTA) as anticoagulant. The umbilical cord was double clamped directly after birth, before the neonate's first breath. Blood samples from the double clamped segment of the umbilical cord were then collected in tubes containing EDTA and immediately frozen at −20°C. Only for the in vitro study was the umbilical cord sample not frozen, but tested immediately. The five full term infants in the in vitro study were not included in the neonatal study, for which red cells from clinically indicated haematocrit measurements were also collected three hours after birth and frozen at −20°C as soon as possible.

Frozen blood samples were gently thawed at room temperature. Glutathione S transferase (EC 2.5.1.18) activity, with 1-chloro-2,4-dinitrobenzene as substrate, was determined after full lysis of the thawed cells with five volumes of ice cold water (10–15 minutes), using the method of Habig and Jacoby,[12] with previously described modifications.[13] Haemoglobin was determined using the method of van Kampen and Zijlstra.[14]

Samples were incubated in a shaking water bath at 80 rpm for one hour at 37°C in increasing concentrations of hydrogen peroxide (concentrations used: 0, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mM $H_2O_2$). All incubations were done in triplicate and the average value of the results was used. For each ml of blood, 100 µl hydrogen peroxide in phosphate buffered saline (PBS: comprising 15 mM $K_2HPO_4$/$KH_2PO_4$, 130 mM NaCl, pH 7.4) were added. The hydrogen peroxide solutions also contained sodium azides (final concentration in the incubates: 0.5 mM) to prevent inactivation of hydrogen peroxide by catalase. After incubation the erythrocytes were washed three times with PBS. Cells were lysed and GST was determined, as described above.

*Table 1 Demographic characteristics of study population*

| | *Healthy, full term (n=8)* | *Sick, premature (n=7)* | *Healthy, premature (n=17)* |
|---|---|---|---|
| Gestational age (weeks) | 39.7 (SE 0.4) | 27.7 (SE 0.7) | 29.2 (SE 0.5) |
| Birthweight (g) | 3456.2 (SE 129.5) | 1071.4 (SE 107.7) | 1169.1 (SE 60.8) |
| Apgar 5 min | 6–10 (median 9) | 6–9 (median 7) | 6–10 (median 9) |
| Umbilical arterial pH | 7.24 (SE 0.01) | 7.14 (SE 0.05) | 7.21 (SE 0.03) |
| Male | 5 | 2 | 9 |
| Female | 3 | 5 | 8 |

1-chloro-2,4-dinitrobenzene (CNDB) and reduced glutathione (GSH) were from Sigma (St Louis, Mo, USA). All other chemicals were of analytical quality. Only filtered, deionised water was used.

The relation between GST in cord blood and gestational age was analysed using linear regression. The Wilcoxon signed rank test was used to test the fall of GST in the first three hours of life. The Kruskal-Wallis test was used to test the difference in GST decrease among the three groups. For the in vitro test, we used the paired *t* test and for the differences in trend we used repeated measurement ANOVA. The Mann-Whitney U test was used for the remaining statistical analyses. A p value of less than 0.05 was considered significant.

## Results

IN VITRO INCUBATION

The difference in the average values in GST activity between adult human blood (4.1 U/g haemoglobin (SE 0.56); n=4) and the cord blood samples (11.2 U/g haemoglobin (SE 1.7); n=5) before incubation with hydrogen peroxide confirmed that GST activity in erythrocytes is much higher in neonates than in adults. On incubation with hydrogen peroxide in increasing concentrations, GST activity was decreased, both in neonatal and adult erythrocytes. The difference between 0.0 and 10.0 mM was significant in both neonatal ($p<0.002$) and adult ($p<0.003$) erythrocytes. There was a significant difference in the fall of GST between adult and neonatal erythrocytes ($p<0.02$). Although the absolute decrease in GST activity was higher in neonates than in adults—3.74 (SE 0.55) and 1.92 (SE 0.22)—respectively, the percentage decrease in GST activity was highest in adults. Part of the GST activity seemed to be highly resistant to hydrogen peroxide. Even at 10 mM initial peroxide concentrations, 67% of the GST activity in neonates and 55% of the activity in adults resisted the incubations (fig 1).

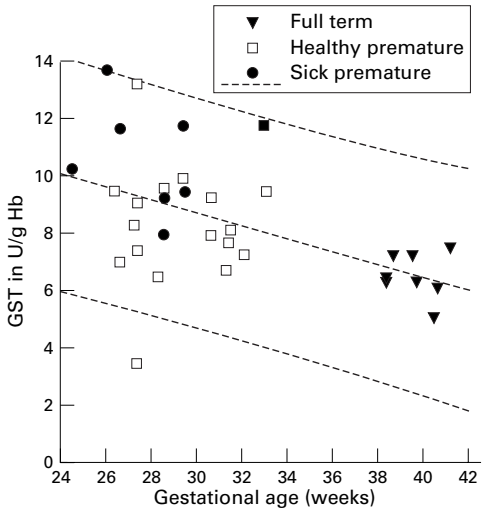


*Figure 2 GST in cord blood plotted against gestational age. Lines represent linear regression (r=0.051) and 95% confidence interval.*

*Table 2 GST activity in U/g Hb in erythrocytes of premature and full term infants in cord blood and at 3 hours postpartum*

| *Infant* | *n* | *Cord blood* | *SE* | *3 hours after birth* | *SE* | *p Value* |
|---|---|---|---|---|---|---|
| Sick, premature | 7 | 10.6 | 0.72 | 8.2 | 0.90 | 0.02 |
| Healthy, premature | 17 | 8.3 | 0.46 | 7.1 | 0.45 | 0.001 |
| Healthy, full term | 8 | 6.7 | 0.28 | 5.5 | 0.49 | 0.02 |

NEONATAL STUDY

Table 1 shows the demographic characteristics of the neonates. The infants who were classified as "sick" differed from their "healthy" premature counterparts with regard to gestational age and birthweight, but this did not reach significance.

GST in cord blood was inversely correlated with gestational age ($p < 0.003$) (fig 2). The mean GST activity in cord blood of the total group was 8.4 (SE 0.39), whereas three hours after birth it was 7.0 (SE 0.36) ($p < 0.0001$).

We compared GST activity in cord blood and three hours after birth in erythrocytes of "sick" premature infants, "healthy" premature infants, and full term neonates. In all three groups GST activity decreased significantly (table 2). The mean decrease for sick premature infants was 2.4 U/g haemoglobin (24%), for healthy premature infants 1.4 U/gHb (16%), and for full term neonates the difference was 1.2 U/gHb (18%). The magnitude of the effect between these three groups did not reach significance ($p=0.18$).

## Discussion

We found that GST activity in cord blood erythrocytes from full term newborns was vulnerable to oxidative stress, similar to GST activity in adult erythrocytes, when treated in vitro with hydrogen peroxide. As in previous reports,[11] we found that GST activity in umbilical cord blood was negatively correlated with gestational age. GST activity three hours after birth was significantly decreased compared with that in cord blood in all three groups tested (sick prematurely born, healthy prematurely born, and healthy full term). There were no significant differences in the magnitude of this effect among the three groups, although the largest decrease was found in the group of "sick" premature infants.

The in vitro results confirm that human erythrocyte GST is vulnerable to hydrogen peroxide treatment, and that this vulnerability already exists in neonatal erythrocytes. On the other hand, more than half the GST activity was very resistant to hydrogen peroxide treatment; a fivefold increase in hydrogen peroxide concentrations from 2 to 10 mM had almost no effect on this remaining activity. This finding is consistent with the 47-Cys modification proposed by Tamai *et al*.[6] This cysteine is near but not in the active centre. As was shown by Ricci *et al*,[15] modification of cysteine affects the catalytic properties of GST, but does not necessarily lead to a full loss of activity. It is also consistent with the finding by Fazi *et al*,[16] that two different forms of human erythrocyte GST-π exist, and that the one which is more heat stable, contains more older erythrocytes. This more heat stable GST could be an oxidised form of Cys-47 that is no longer susceptible to heat denaturation.

Whatever the mechanism involved, our in vitro results showed that GST inactivation in erythrocytes could be a valuable indicator of oxidative stress in neonates. Although the hydrogen peroxide concentrations used in vitro were relatively high compared with what might occur in vivo, differences in exposure time could account for this. The actual exposure time in the in vitro experiments was even shorter than the incubation time because the hydrogen peroxide is rapidly inactivated by glutathione peroxidase[17] (catalase was inhibited in these experiments). Kinetic experiments showed that most of the GST inactivation does indeed occur during the first few minutes of the incubation (data not shown).

The results of the measurements of red cell GST activity in premature and term infants in the neonatal study confirmed that GST activity in erythrocytes is negatively correlated with increasing gestational age, as shown by Ripalda *et al*.[11] Surprisingly, however, we found that GST activity in cord blood samples from the term neonates used in the in vitro study, was higher than that in the full term infants, and even the premature infants in the neonatal study. The higher GST activity in the cord blood, used in the in vitro study, is probably explained by differences in treatment between these samples and the samples used in the in vivo study.

The in vitro study confirmed the sensitivity of GST to hydrogen peroxide treatment. The high oxygen challenge that occurs at birth might lead to increased formation of reactive oxygen species. The interaction between haemoglobin and oxygen will, for instance, increase superoxide formation,[18] which, apart from doing some damage itself, will be converted to hydrogen peroxide. The interaction of HbF with oxygen can generate greater amounts of superoxide, hydrogen peroxide, and hydroxy radicals than that of HbA.[19] Other factors such as a membrane lipid composition which is more vulnerable to lipid peroxidation, low concentrations of vitamin E and some antioxidant enzymes, may also contribute to this phenomenon. This increased formation of reactive oxygen species is, in our view, the most likely explanation for the decreases in GST activity three hours after birth. The more pronounced decrease in GST activity in sick premature infants could be the result of less well developed antioxidant systems in this group with the lowest gestational age, or it might be the result of their condition and treatment. This is consistent with the results of Nycyk *et al*,[20] who found that high peak pentane exhalation, a marker of lipid peroxidation, was associated with low gestational age, mortality, intraventricular haemorrhage and retinopathy of prematurity.

We conclude that the determination of changes in erythrocyte GST activity is a promising indicator of oxidative stress conditions that occur at birth. Measurement of GST

activity might be useful for the evaluation of prophylactic treatment in trials of antioxidant strategies.


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